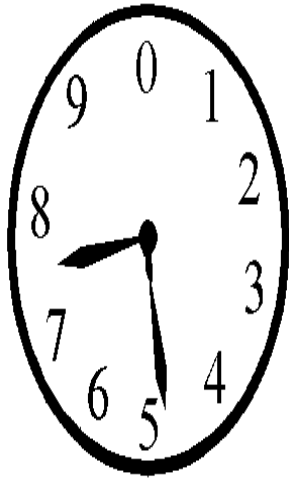


# Prioritizing Our Validation Efforts



Andy Hoofnagle, MD PhD  
University of Washington  
Friday the 13th

# Protein Biomarkers

***Why quantify proteins in complex mixtures?***

Basic science

Discovery

Select patients for clinical trials

Toxicity

Efficacy

Diagnosis

Prognosis

Therapeutic management

*Because it is fun*

# Protein Biomarkers

## **Quality control of multi-protein assays**

*Like walking quickly, carrying a platter with a bunch of different stacks of vegetables...*

## **Single proteins**

- Relatively straight-forward

- Many relevant technologies

- Defined development, validation, and quality control

## **Multi-protein panels**

- Many different assays or

- One multiplexed assay

- Development of each component

- Parametric or non-parametric combinations

- Validation may be difficult to conceive of

- Quality control...

# Quality Control of Multi-Protein Panels



# Experiments

## Basic science

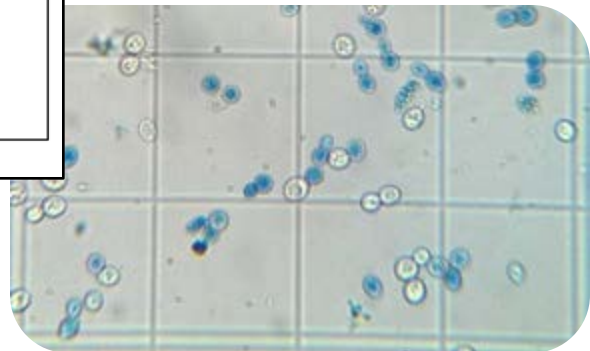
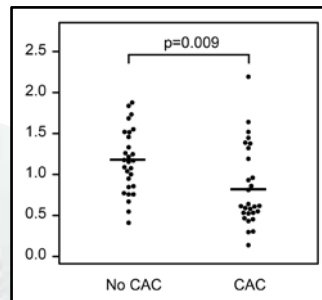
Cells

Animals

A few human samples

Discovery

*Expect trends to repeat...not necessarily the numbers*



# Experiments

## Human subjects, retrospective

Discovery

Verification

Qualification

May not need IRB approval

*Freezer diving*



# Experiments

**Patients, prospective clinical trials**

Diagnosis

Prognosis

Management

Need IRB approval for these studies

These assays need to be fully validated

**These assays need to be fully validated**

*Using novel biomarkers in patient care*



# Validation

*A sincere attempt to demonstrate the robustness of a system*

***Think about the parts of an immunoMRM assay...***

Phlebotomy

Blood draw tube

Storage

Pipetting, tips, tubes, plates, vials

Internal standards

Denaturation, reduction, and alkylation

Digestion

Magnetic beads

Antibody

Washing and elution

Autosampler

HPLC pumps

Chromatographic column

Mass Spectrometer



# Validation

## Regulated

Rigorously define performance characteristics  
Suggested acceptability—best practices  
Documentation

*Transparency not really a component*



Advancing Transfusion and  
Cellular Therapies Worldwide



# Validation

## **Unregulated**

Publishing guidelines

Funding agency guidelines

Peer reviewers

Training

*Transparency must be a fundamental tenet*

# Tiers of Assay Quality

Tier and Areas of Application	Degree of Analytical Validation	Labeled Internal Standards	Reference Standards	Specificity	Precision	Quantitative Accuracy	Repeat-ability	Comments and Suggested References
<b>Tier 1</b> Clinical bioanalysis/ diagnostic laboratory test; single analyte or small numbers of analytes	High, including batch-to-batch QC	Yes, for every analyte	Yes	High	High (typically <20-25% CV achieved)	Defining accuracy is a goal; true accuracy difficult to demonstrate.	High	Precise, quantitative assays; established, high performance; may need comply with FDA and CLIA guidance depending on use of assay  Refs. 30, 41, 42, 53
<b>Tier 2</b> Research use assays for quantifying proteins, peptides, and post-translational modifications; 10's to 100's of analytes	Moderate-to-high	Yes, for every analyte	Limited use	High	Moderate-to-high (typically <20-35% CV achieved)	Not applicable	High	Precise, relative quantitative assays; established performance; suitable for verification  Refs. 30, 31, 36, 37, 40, 51, 70, 71
<b>Tier 3</b> Exploratory studies; 10's to 100's of analytes	Low-to-moderate	None-to-limited	No	Moderate-to-high	Low-to-moderate: similar to label-free discovery	Not applicable	Moderate-to-high	Discovery in a targeted mode; performance not defined; results require further verification using quantitative techniques  Refs. 36, 37, 86-89

# Tiers of MRM Assay Quality



Tier 1	Tier 2	Tier 3
Clinical diagnosis	Research	Discovery
Internal standards	Internal standards	
Highly precise	Precise	Less precise
QC procedures		
Values repeatable	Trends repeatable	Large magnitude changes
One to a few analytes	10s-100s of analytes	10s-100s of analytes

*Fit-for-purpose approach*

*Carr, MCP, 2014*

# Replicating Tier 2: Peptides

## Experiment 1: *Response Curve*

- Development of multipoint response curve (1 blank and a minimum of 6 concentration points).
- Samples prepared in digested matrix background (i.e. plasma, tissue, cells, etc).
- Used for the determination of LOD, LLOQ and linearity.
- Multiple replicates analyzed.

Preliminary validation of peptide quantification in complex mixtures

## Experiment 2: *Mini-validation of Repeatability*

- Examines intra- and inter-assay variability.
- Uses the LLOQ from Experiment 1 from which 3 concentrations (Low, Medium and High) are used to assess repeatability.
- 3 replicates processed and measured on 5 different days.

## Experiment 3: *Selectivity*

- Examines the response of a peptide in six different biological replicates of the matrix.
- Replicates analyzed with no spike and ½ the Medium and Medium concentrations defined in Experiment 2.

## Experiment 4: *Stability*

- Examines the stability of a peptide spiked into a background matrix
- Stability assessed based on peak area variability following:
  - different storage conditions (4C and -70C) over time.
  - freeze-thaw cycles
- Variability compared to data collected from Experiment 2.

## Experiment 5: *Reproducible Detection of Endogenous Analyte*

- Representative sample containing endogenous analyte is digested 5 times on each of 5 days.
- Examines intra- and inter-assay variability of the entire assay workflow, including digestion.

CPTAC  
Assay Development Working Group

# NCI Assay Portal



National Cancer Institute

at the National Institutes of Health | [www.cancer.gov](http://www.cancer.gov)



OFFICE OF CANCER CLINICAL  
PROTEOMICS RESEARCH  
Assay Portal

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Available Assays About

CPTAC Home

## Assay Portal



### Search the Assay Database by:

[x Clear All Filters](#)

Search:

Showing 1 to 50 of 456 entries

Show 50  entries

Show / hide columns

Download CSV

KEGG Pathways

Select

Data Source:  
[KEGG](#)

Find assays to  
proteins encoded in  
a specific  
chromosomal region

Chromosome  
Number

Include All

← Previous 1 2 3 4 5 Next →

Proteins and peptides for which assays are available	Submitting Laboratory	Modification	Assay Type	Matrix	CPTAC ID
AARS - UniProt Accession ID: P49588					
AVFDETYDPVR	Fred Hutchinson Cancer Research Center	unmodified	direct MRM	cell line lysate pool	CPTAC-1
TITVALADGGRPDNTGR	Fred Hutchinson Cancer Research Center	unmodified	direct MRM	cell line lysate pool	CPTAC-2

CPTAC Assay Development Working Group

# Translating Tier 2: Proteins

Validation		Experiment
Reproducibility		5x5 experiment
Peptide degradation	Spike IS peptides before/after digestion	
Linearity	Mix pools together	
LLOQ	Dilution experiments of one or more pools	
Interferences	Add potential interferences to pools	
Stability	Stress pools before and after sample prep	

Validating protein quantification for publication

# Guidance Documents: CLIA & CLSI

Performance specification	References
Precision	EP05, EP29
Linearity	EP06
Analytical sensitivity	EP17
Specificity and interferences	EP07, EP14
Accuracy and method comparison	EP09
Multiple instruments	EP31
Carryover	EP10
Quality control	EP28

Validating protein quantification for the care of patients



# Accuracy

**The accuracy of a measurement is the closeness of agreement between the test result and the true result**

## **Precision**

- Within batch

- Between batch

- Between laboratory

## **Bias**

- Calibration materials

- Sample-specific matrix effects

*How similar will a result be if measured in Seattle on Monday and in London on Wednesday? Is it right?*

# Harmonization

**Trying to make results as similar as possible**

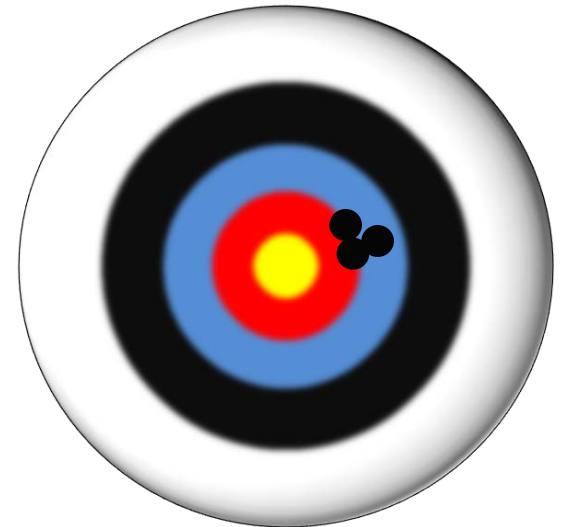
## **Examples**

- Two different platforms in the same laboratory
- The same platform in different laboratories
- Two different platforms in two different laboratories

## **Methods**

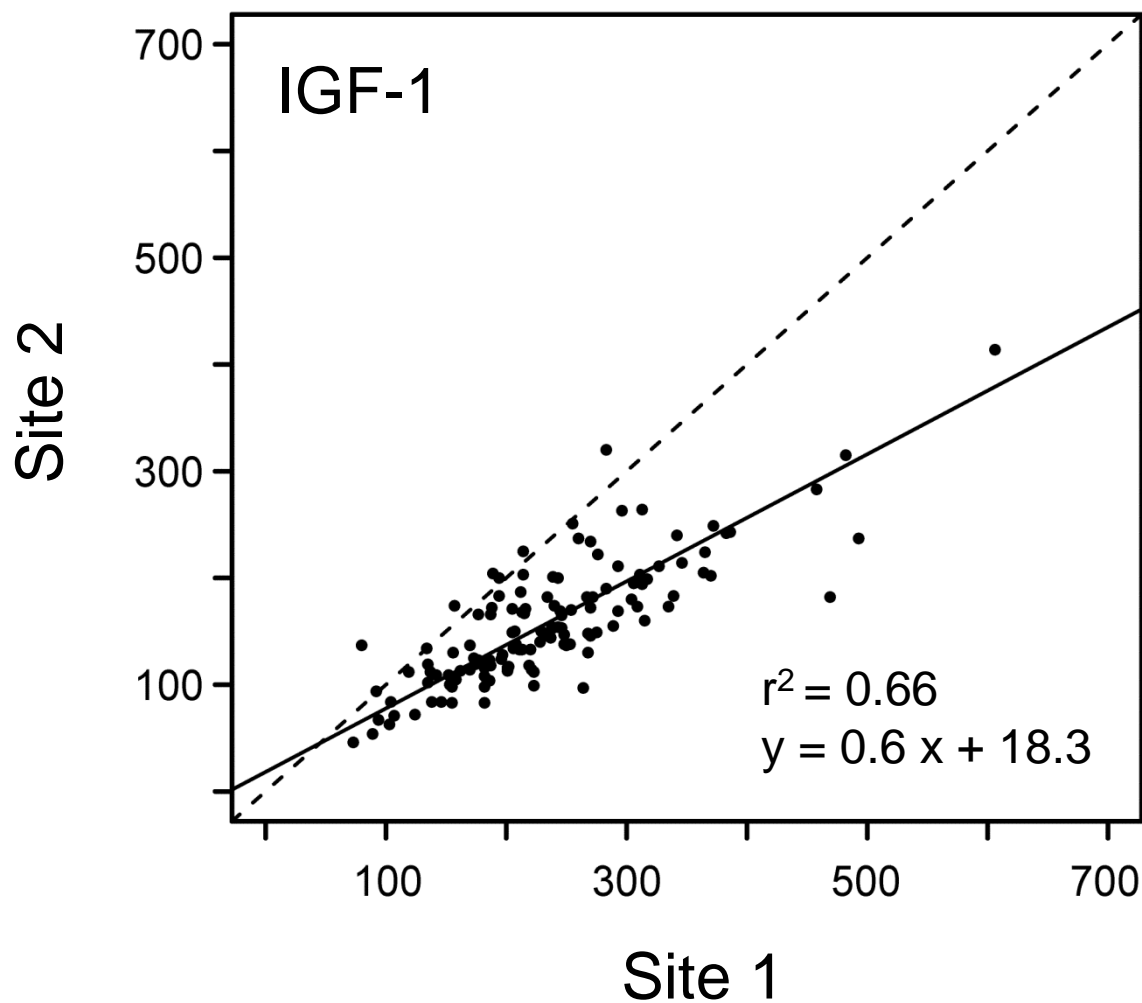
- Non-certified reference material
- Cooperation within a consortium
- Recalibration to a single central laboratory

*Improving between-platform concordance*  
*Reducing between-laboratory variability*  
*Not necessarily the true answer*



# This is Not Harmonization

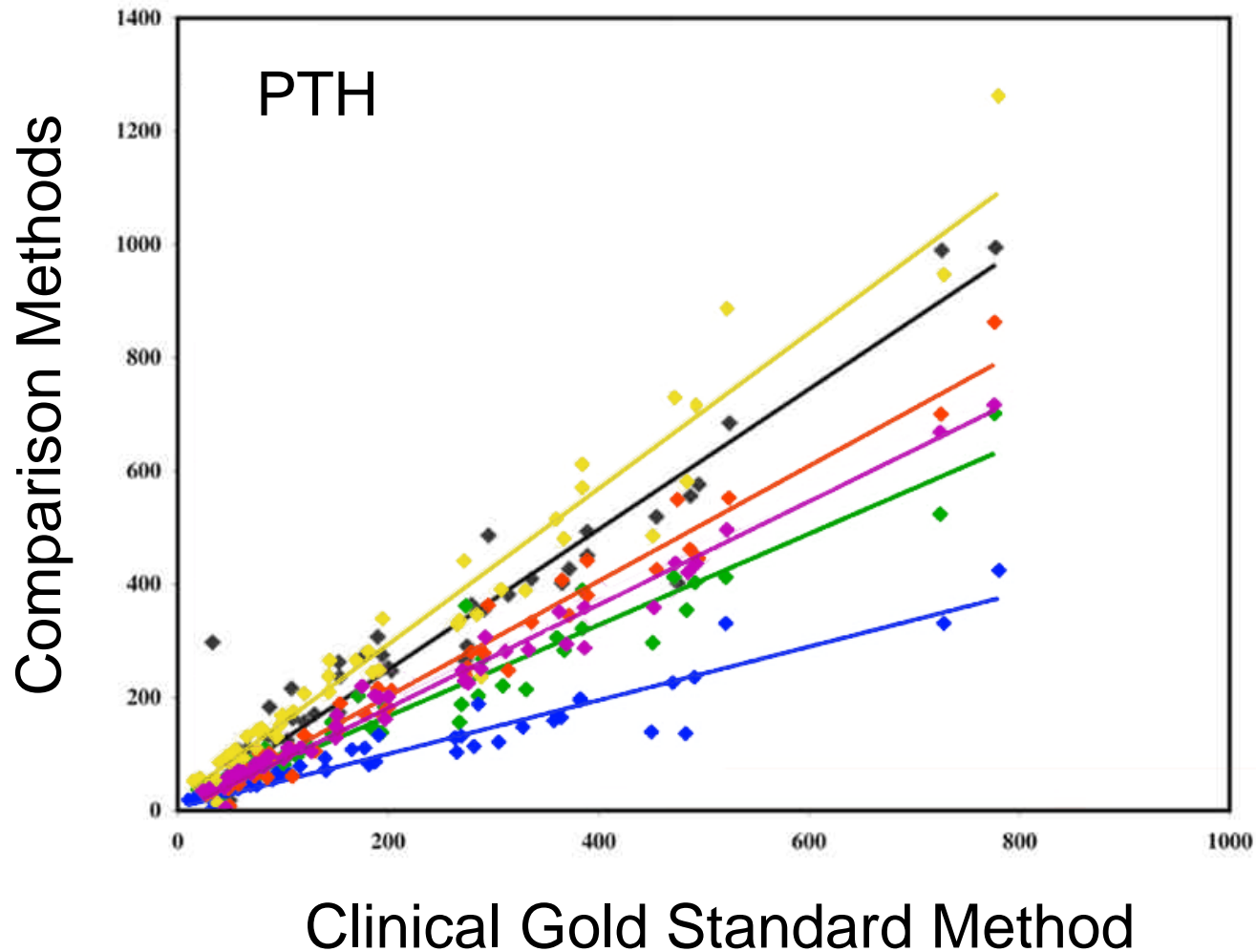
*One FDA-approved Immunoassay, Two Laboratories*



*Cox, ClinChem, 2014*

# This is Not Uncommon

*Seven FDA-approved Immunoassays, One Laboratory*



# Standardization

**Trying to make results from all platforms in all laboratories give the same true result**

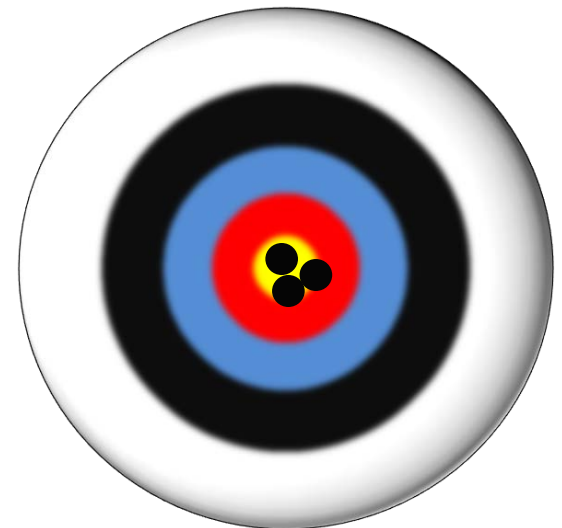
## **Methods**

- Reference method procedures
- Certified reference materials
- Traceable calibration materials

## **Examples**

- Vitamin D Standardization Program
- Hormone Standardization Program
- Hemoglobin A1c Standardization (NGSP)

*Pre-established acceptance criteria*  
*Commutable materials*



# Proficiency Testing

**Keeping it real over time**

## **Methods**

Spiked synthetic matrix

Stabilized human samples

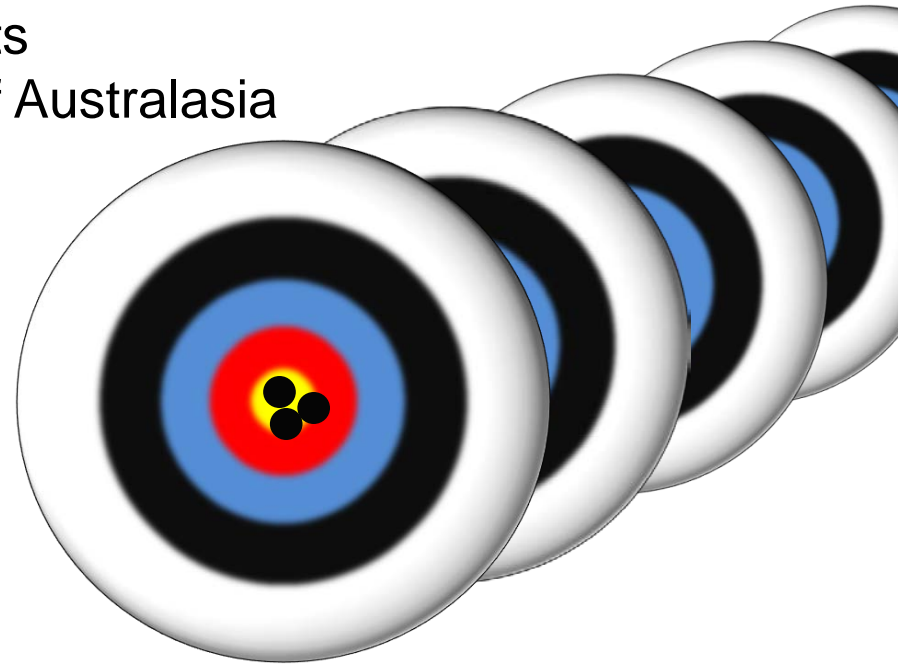
Minimally-processed native human samples

## **Examples**

College of American Pathologists

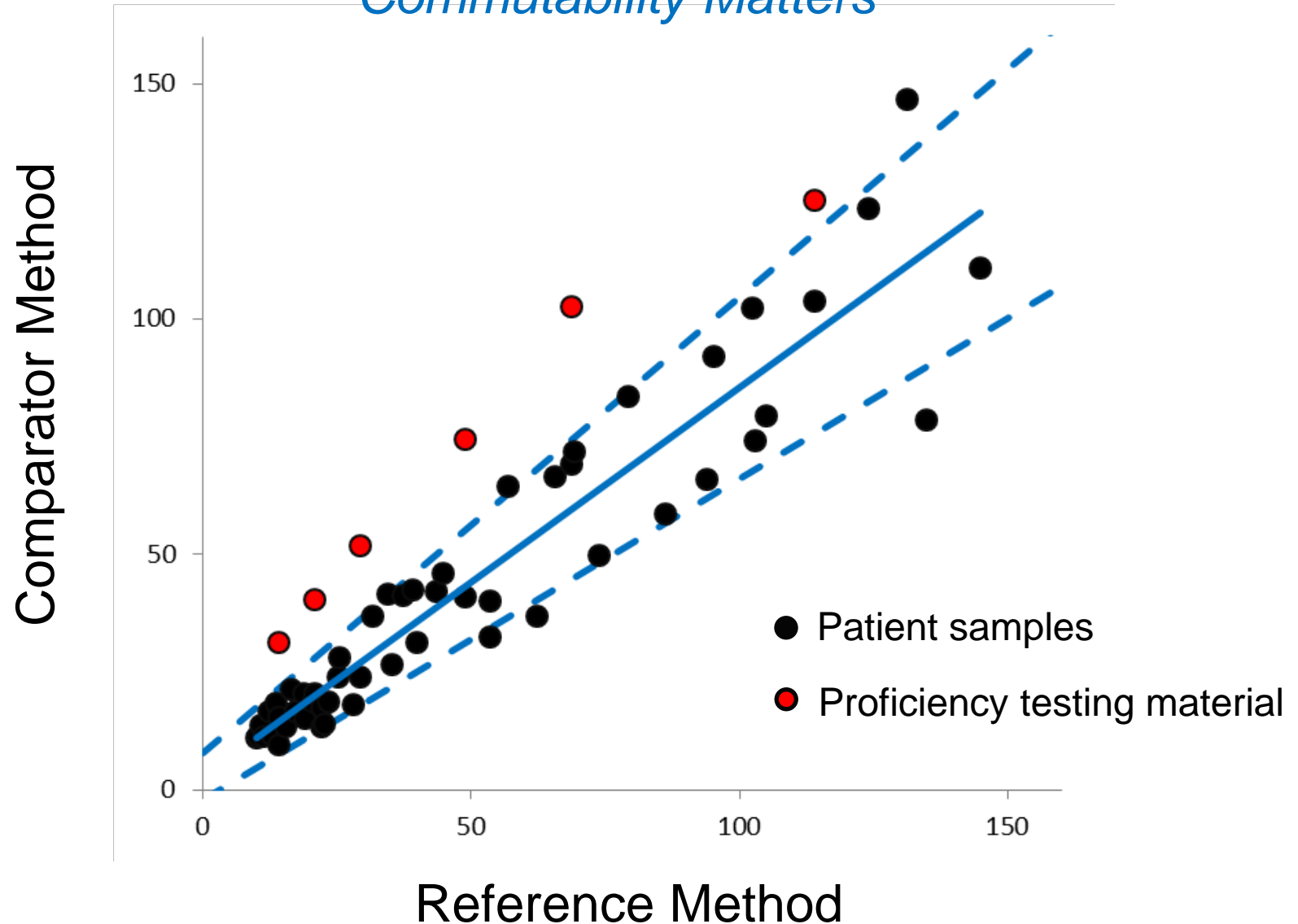
Royal College of Pathologists of Australasia

DEQAS



# Proficiency Testing

*Commutability Matters*



# Proficiency Testing

## *Accuracy Based*

### 25-OH Vitamin D, total – ng/mL

	METHOD	NO. LABS	MEAN	S.D.	C.V.	MEDIAN	LOW	HIGH
							VALUE	VALUE
ABVD-04	Abbott Architect i System	69	18.87	1.28	6.8	19.0	15.4	21.3
	Diasorin Liaison	56	16.43	1.57	9.5	16.0	13.0	20.5
	Immunodiagnostic Systems (IDS) EIA	11	21.98	5.50	25.0	23.0	10.8	31.3
	Immunodiagnostic Systems (IDS) iSYS	9	-	-	-	23.0	20.5	29.0
	Liquid Chromatography-Mass Spectrometry- Mass Spectrometry (LC-MS-MS)	68	23.55	2.47	10.5	23.5	17.0	29.5
	Roche cobas e411/electsys	18	14.19	1.23	8.6	13.9	12.0	17.2
	Roche cobas e600 series/e170	59	14.01	1.36	9.7	14.1	11.0	17.5
	Siemens Diagnostics ADVIA Centaur, Centaur XP	93	19.95	2.95	14.8	19.4	11.7	27.8
	<b>All Methods</b>	<b>404</b>	<b>18.71</b>	<b>4.05</b>	<b>21.7</b>	<b>18.5</b>	<b>9.0</b>	<b>31.3</b>
	<b>Reference Target*</b>		<b>21.95</b>					

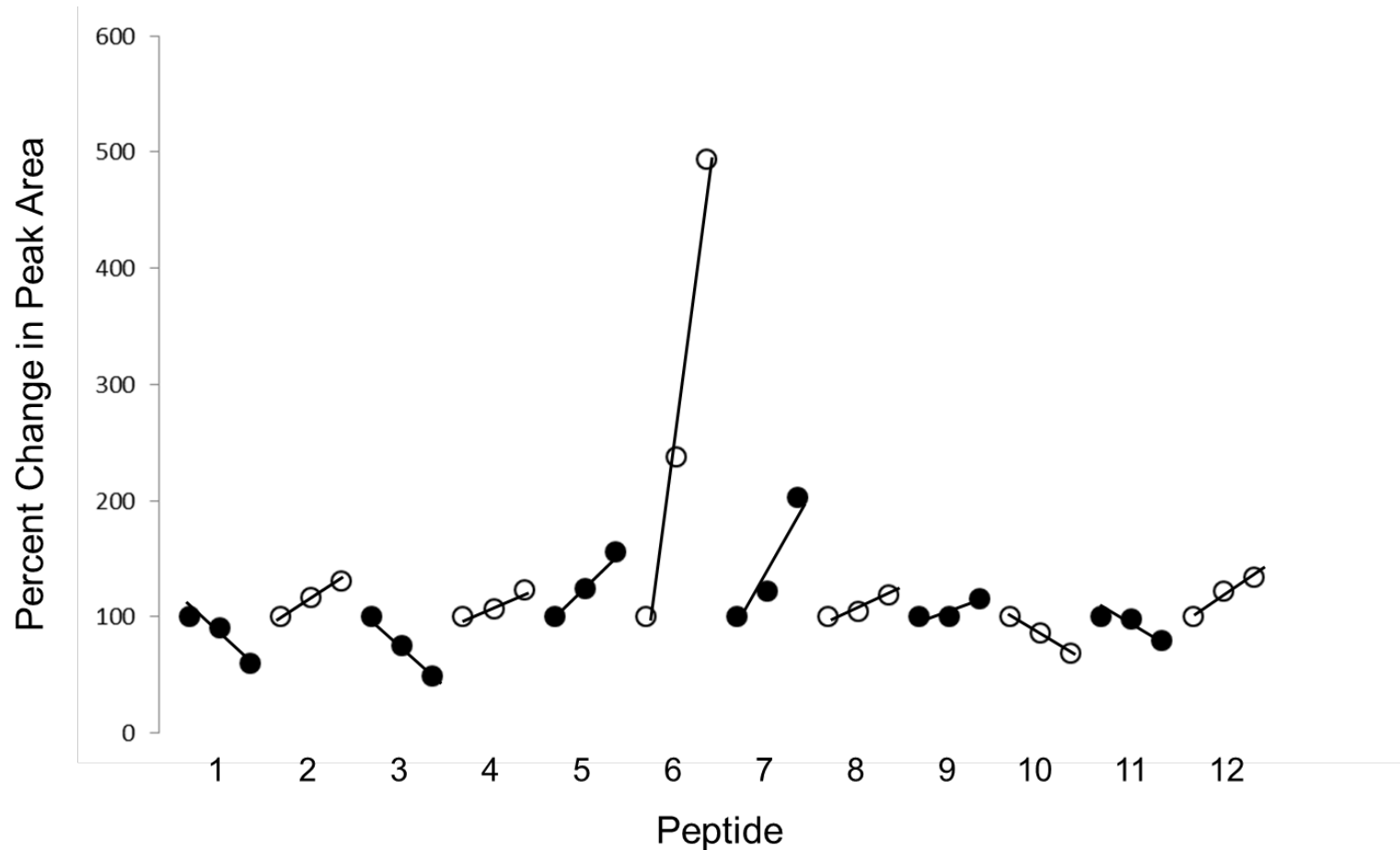
	METHOD	NO. LABS	MEAN	S.D.	C.V.	MEDIAN	LOW	HIGH
							VALUE	VALUE
	Abbott Architect i System	69	34.33	1.94	5.7	34.0	30.0	38.5
	Diasorin Liaison	56	31.80	2.76	8.7	31.6	26.9	39.9
	Immunodiagnostic Systems (IDS) EIA	11	38.11	7.79	20.4	36.6	20.7	49.1
	Immunodiagnostic Systems (IDS) iSYS	8	-	-	-	41.3	34.7	44.0

*CAP is moving toward accuracy based programs...a great step forward*



Does calibration have a role in research  
proteomics assays?

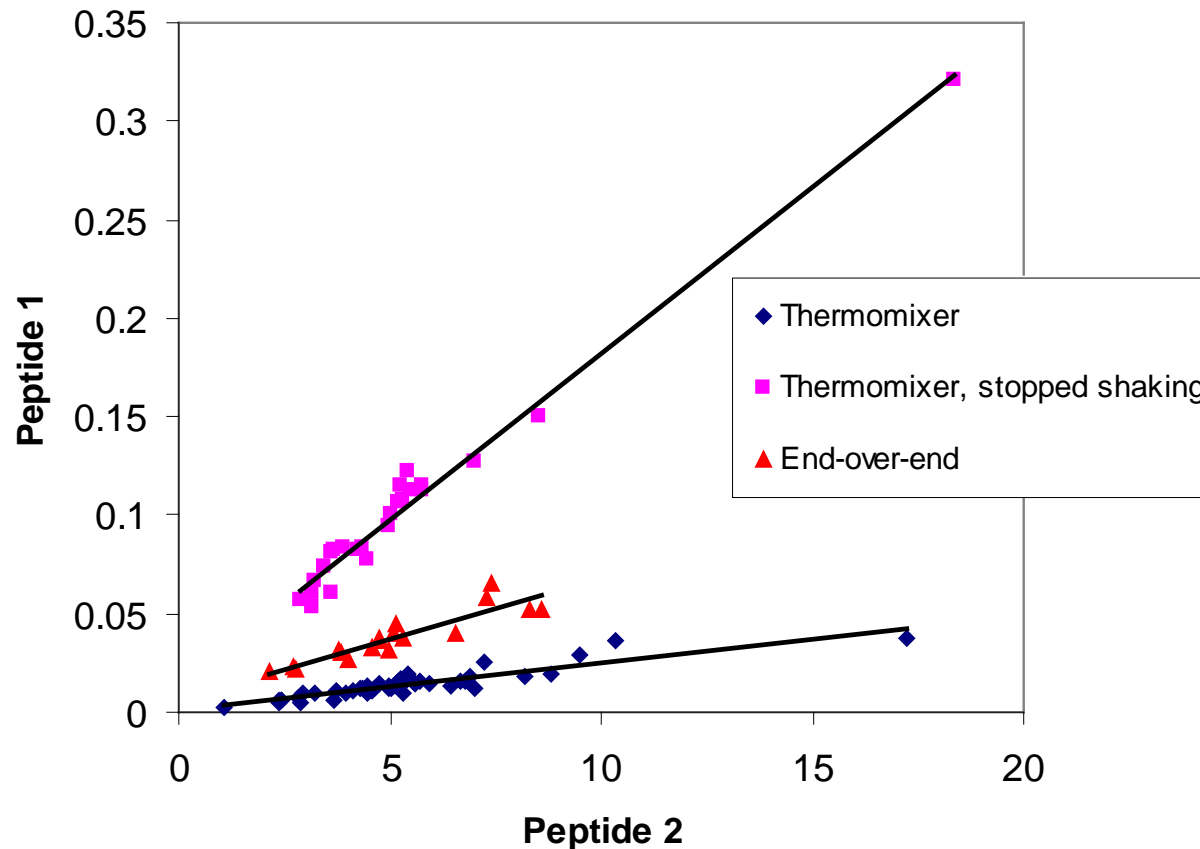
# Variability of Peptide Liberation



*Vary the amount of trypsin (50%-200%)*  
*Very different results for each peptide*

# Variability of Peptide Liberation

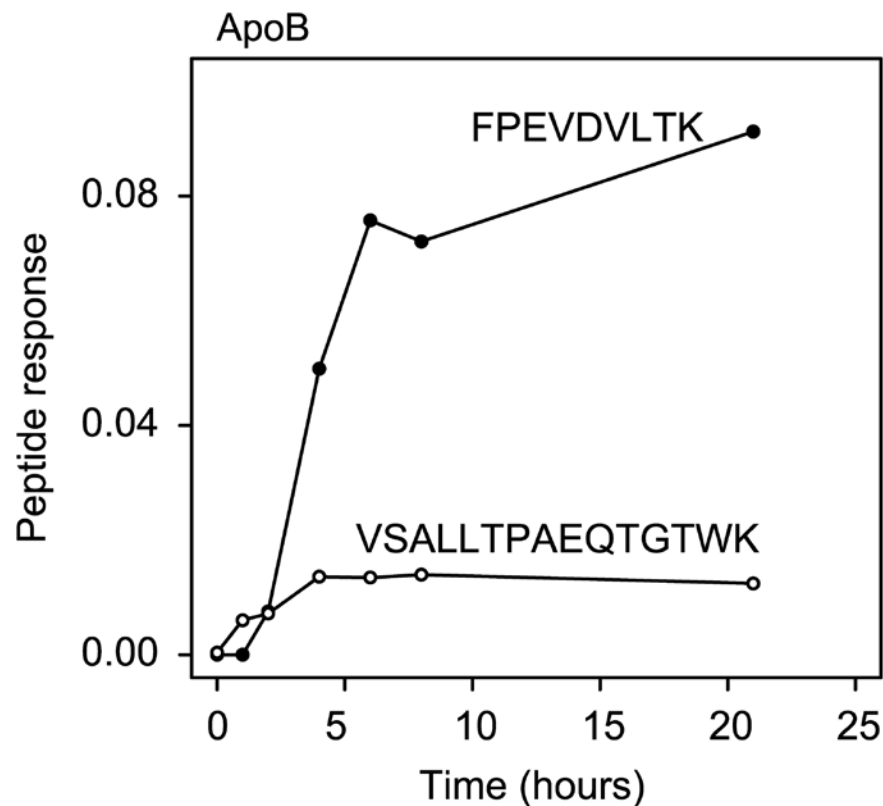
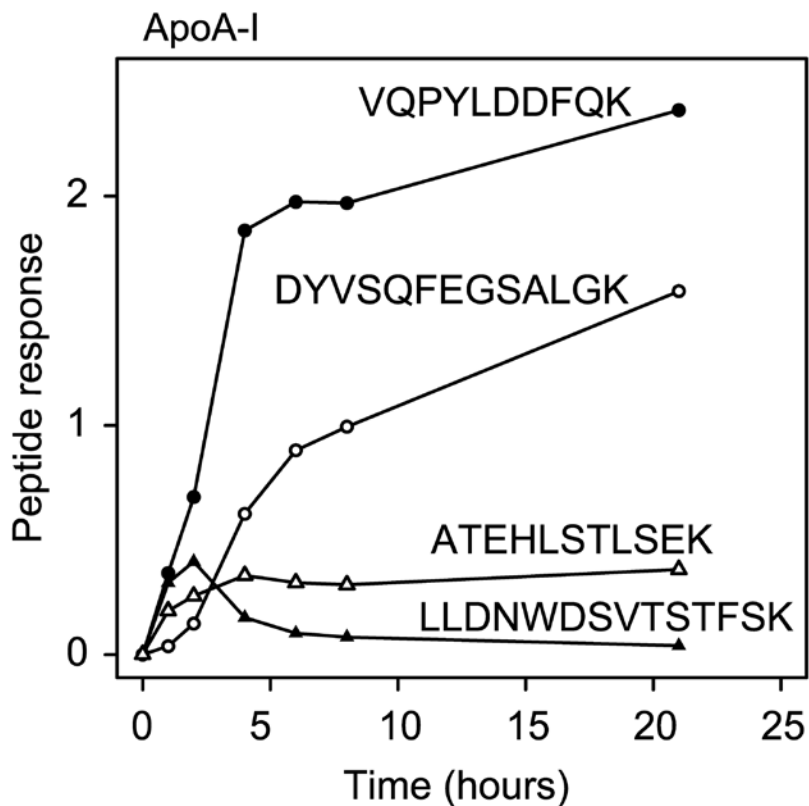
*Mechanism of agitation matters*



*How can two peptides from the same protein be so different?*

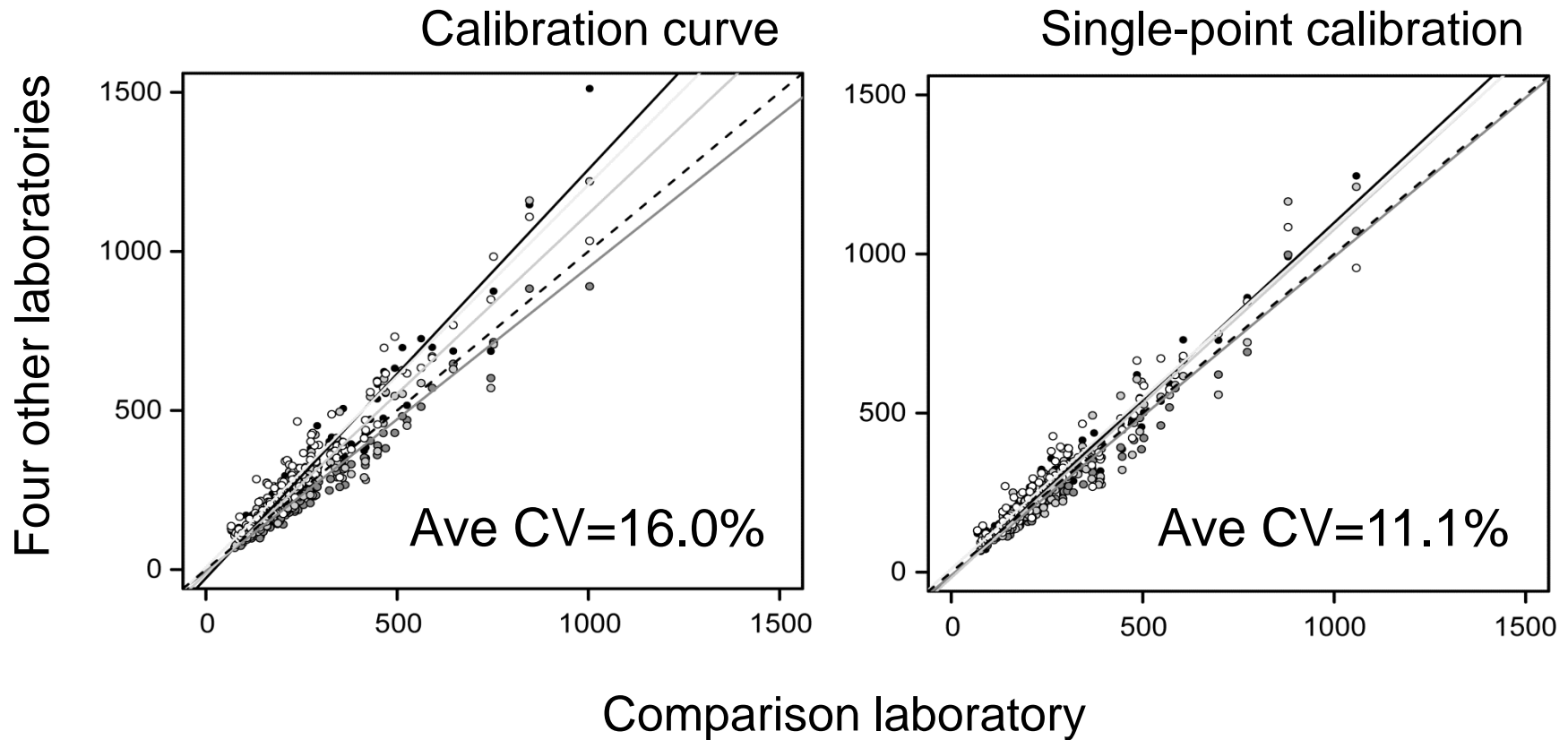
# Variability of Peptide Liberation

*Are we ever done?*



# Calibration Improves Concordance

*Between-laboratory repeatability*



# Conclusions

Calibration is the cornerstone of repeatability

Validation can be fit-for-purpose

Effective translation requires adequate validation

Transparency is pivotal for generalization

Life after validation...

- ...harmonization

- standardization

- proficiency testing

# Conclusions

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# Acknowledgements

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